Crosstalk between intracellular zinc rises and reactive oxygen species accumulation in chemical ischemia

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Running title: Crosstalk between zinc and mitochondrial ROS

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ABSTRACT

Both zinc (Zn²⁺) and reactive oxygen species (ROS) have been shown to accumulate during hypoxic-ischemic stress and play important roles in pathological processes. To understand the crosstalk between two of them, here we studied Zn²⁺ and ROS accumulations by employing fluorescent probes in HeLa cells to further the understanding of the cause and effect relationship of these two important cellular signaling during chemical-ischemia, stimulated by oxygen and glucose deprivation (OGD). We observed two Zn²⁺ rises that were divided into four phases in the course of 30 minutes of OGD. The first Zn²⁺ rise was a transient, which was followed by a latent phase during which Zn²⁺ levels recovered, however kept above a basal level in most cells. The final phase was the second Zn²⁺ rise that reached a sustained plateau called Zn²⁺ overload. Zn²⁺ rises were not observed when Zn²⁺ was removed by TPEN (a Zn²⁺ chelator) or thapsigargin (depleting Zn²⁺ from intracellular stores) treatment, indicating that Zn²⁺ were from intracellular storages. Damaging mitochondria with FCCP significantly reduced the second Zn²⁺ rise, indicating that the mitochondrial Zn²⁺ accumulation contributes to Zn²⁺ overload. We also detected two OGD-induced ROS rises. Two Zn²⁺ rises preceded two ROS rises. Removal of Zn²⁺ reduced or delayed OGD- and FCCP-induced ROS generation, indicating that Zn²⁺ contributes to mitochondrial ROS generation. There was a Zn²⁺-induced increase in functional component of NADPH oxidase, p47phox, thus suggesting that NADPH oxidase which may mediate Zn²⁺-induced ROS accumulation. We suggest a new mechanism of crosstalk between Zn²⁺ and mitochondrial ROS through positive feedback processes that eventually causes excessive free Zn²⁺ and ROS accumulations during the course of ischemic stress.

Zinc (Zn²⁺) is an important element in physiology, and is recognized to play a role in hypoxia/ischemia-induced cytotoxicity as Zn²⁺ accumulation precedes cell death (39, 50, 69). The removal of Zn²⁺ with a chelator has been shown to reduce cytotoxicity (26, 40, 62). Reactive oxygen species (ROS) produced by mitochondria have been of interest for many years due to their involvement in cellular signaling at moderate levels and in pathological mechanisms of cell death at high levels (17, 54, 68, 75). Many studies on hypoxia/ischemia-induced cytotoxicity have focused on ROS because hypoxic exposures induce oxidative stress and excessive ROS accumulation, which subsequently damages cells. Given its importance in cell stress signaling, the sources, mechanisms, and time course of ROS generation during ischemia and re-oxygenation continue to receive intensive investigation. Intracellular Zn²⁺ accumulation has been associated with cytotoxicity and has been observed shortly after the onset of
ischemia. We recently showed that hypoxia exposure induced a rapid Zn$^{2+}$ transient (Zn$^{2+}$ wave) that preceded mitochondrial ROS induction and accumulation (66). We are thus compelled to further study the possible crosstalk between these two prominent signaling systems during ischemia like condition of oxygen and glucose deprivation (OGD), which may lead to better understanding of the regulation mechanism between the two important events.

Total Zn$^{2+}$ concentration in human cells is in the range of several hundred micromolar (µM) with most Zn$^{2+}$ being bound to proteins or sequestered into intracellular organelles (44). Zn$^{2+}$ is tightly regulated in a healthy cell where labile or free Zn$^{2+}$ is maintained in picomolar (pM) range, because free Zn$^{2+}$ is toxic to the cells (26, 40, 62, 65). Intracellular compartments and Zn$^{2+}$ binding proteins are involved in keeping Zn$^{2+}$ homeostasis. Among these, mitochondria are the key intracellular organelle for buffering Zn$^{2+}$ levels in neurons (62). Studies demonstrated that Zn$^{2+}$ overload in mitochondria would induce multiple mitochondrial injuries (28, 50, 63) and activate mitochondrial-mediated pro-apoptotic factors (25, 35, 61). Zn$^{2+}$ is also reported to activate mitochondrial outer membrane channels (6, 31) and cytochrome C discharge from mitochondria (12, 24). Studies also show that other organelle storages such as endoplasmic reticulum (ER) (42, 70) contain free and mobile Zn$^{2+}$ pool. Together with ubiquitous metallothioneins (MT), they modulate Zn$^{2+}$ homeostasis by serving as either sources or sinks of Zn$^{2+}$.

The understanding of contributing factors in ROS production have been evolving in recent years, with mitochondria, xanthine oxidase and NADPH oxidase playing an important role in ROS generation (5, 78). For example, the activity of NADPH oxidase and ROS production can be seen increasing with the duration of hypoxia (10) and mitochondria are in communication with NADPH oxidase produced ROS (2, 18). Recent studies have highlighted the notion that Zn$^{2+}$ and ROS signaling systems are intimately integrated such that Zn$^{2+}$-dependent regulation of components of ROS homeostasis might influence intracellular redox balance, and vice versa. Zn$^{2+}$-induced cell death is accompanied by increased levels of ROS, and is attenuated by various antioxidative measures. On one hand, a number of ROS-generating and antioxidant systems of living cells have been shown to be Zn$^{2+}$-dependent (62, 65). If both play key roles in regulating cellular stress responses, than the question is – what is the interaction or crosstalk between Zn$^{2+}$ and ROS? The objective of the present study was to identify the fundamental processes that determine Zn$^{2+}$ and ROS accumulation, to explore the cause and effect relationship between Zn$^{2+}$ rises and mitochondrial ROS production in cells during OGD. Based on the data presented here, we proposed a novel positive feedback mechanism of crosstalk between these two intracellular signals.

RESULTS

OGD-induced [Zn$^{2+}$], rises and their relationships with [ROS]$_{mi}$ production —To induce hypoxia like response in live cells, an oxygen scavenger and a reducing agent sodium dithionite was used. This method of “chemical” hypoxia was widely used to rapidly induce low oxygen environment with reliability (23, 29, 56, 60, 77). Others used this method of hypoxia induction paralleled with oxygen deprivation (like we did in brain slice experiments, discussed further) (41, 43). To simulate ischemia like condition, in addition to chemical hypoxia, we removed glucose from the solutions as well, inducing a state of oxygen and glucose deprivation (OGD). To measure the changes in intracellular Zn$^{2+}$ concentration ([Zn$^{2+}$]) during the course of OGD, cells were labeled with intracellular fluorescent free Zn$^{2+}$ indicator FluoZin-3, AM (1µM) with $K_{d}$ about 15nM. Basal cytosolic Zn$^{2+}$ distribution with FluoZin-3, AM appeared generally very faint although in some cells a brighter Zn$^{2+}$ fluorescence was observed around nucleus. Nucleus had no detectible free Zn$^{2+}$ and appeared as a dark oval structure in the middle of the cell. Cells in OGD treatment were continuously bathed in the OGD buffer. Changes in fluorescence intensity indicated changes in intracellular free Zn$^{2+}$ concentration. Note, that the extracellular solutions during the OGD experiments did not contain detectible amount of free Zn$^{2+}$. We observed two Zn$^{2+}$ elevations in the course of 30 minutes of hypoxic treatment (Figure 1A&B). On the other hand, cells in control test were continuously bathed in physiological buffer and yielded no
significant change in the fluorescence. The time course of intracellular Zn\(^{2+}\) elevations can be divided into four phases (Figure 1A-C). Phase I is the ascending part of the first Zn\(^{2+}\) rise (Zn\(^{2+}\) transient) observed and is defined by a sharp increase in [Zn\(^{2+}\)], beginning as early as 60 seconds after onset of OGD treatment, and continued to ascend with a sharp slope for 90 seconds. Phase I was quickly followed by Phase II which is the descending part of cytosolic Zn\(^{2+}\) transient and characterized by Zn\(^{2+}\) returning toward basal level (Figure 1A). Because cells were continuously bathed in OGD buffer, the majority of examined cells (20/33, 61%) had rather a short phase II and Zn\(^{2+}\) did not return to basal level but maintained a sustained increase in [Zn\(^{2+}\)], (Figure 1A-1). There were about 40% cells in which Zn\(^{2+}\) transient returned close to the baseline and the phase II lasted for about two minutes (Figure 1A-2). The third phase (III) is a latent phase, during which Zn\(^{2+}\) transient gradually elevated above basal levels (Figure 1A). The duration of latent phase was variable but generally ranging around 10 minutes. The latent phase was followed by phase IV which was the second sharp Zn\(^{2+}\) elevation characterized by a substantial increase in [Zn\(^{2+}\)], to a sustained plateau, which we have also termed Zn\(^{2+}\) overload. Phase IV or Zn\(^{2+}\) overload was observed in all examined cells (Figure 1A) and onset of the overload started consistently around 19-22 minutes of OGD exposure. Overall, its amplitude was about twice as large as the amplitude of phase I. It did not exhibit a wave like transient and only had an ascending component that continued for about 4 minutes before reaching a plateau, where there was continued increase in [Zn\(^{2+}\)], throughout observation time.

The OGD-induced increases in [Zn\(^{2+}\)], was compared to mitochondrial ROS accumulation ([ROS]\(_{\text{mito}}\)), to explore possible temporal relationship of these two important phenomena during ischemia like conditions of OGD. Mitochondrial superoxide indicator MitoSOX Red (5µM) was used to study ROS accumulation in mitochondria. The probe’s distribution resembled mitochondrial distribution, described previously, where mitochondria was labeled with mitochondria specific fluorescent dye MitoFluor Red (42). When cells were exposed to OGD under the same condition as in Zn\(^{2+}\) fluorescence labeled cells, [ROS]\(_{\text{mito}}\) started to rapidly rise after 5 minutes of exposure and continued to rise for 4 minutes, after which the ROS levels plateaued and remained elevated until 22-23 minutes of exposure (Figure 1C). The plateau resembled the latent phase seen in OGD-induce Zn\(^{2+}\) elevation, and was followed by the second ROS increase. During the period of delayed Zn\(^{2+}\) overload, the mitochondrial ROS sharply increased and continued to increase during the remaining observation. To show the temporal relationship of these two signals, Zn\(^{2+}\) fluorescence and mitochondrial ROS fluorescence were plotted together on one plot (Figure 1C). The first Zn\(^{2+}\) elevation (Zn\(^{2+}\) transient) precedes the beginning of [ROS]\(_{\text{mito}}\) increase that corresponds with Zn\(^{2+}\) transient on the late phase II. While steady and sustained ROS production is parallel to latent phase and is prior to the second sharp Zn\(^{2+}\) elevation, the latter precedes the second sharp ROS increase.

**Intracellular sources of OGD-induced [Zn\(^{2+}\)], rises depended on TPEN, TG and FCCP sensitive stores** - To explore the possible contributions of intracellular Zn\(^{2+}\) sources to OGD-induced Zn\(^{2+}\) rises, we treated cells with TPEN, a membrane permeable Zn\(^{2+}\) chelator. TPEN (35 µM) was applied 5 minutes before the application of OGD buffer and was also included in OGD buffer throughout the hypoxic treatment. As shown in Figure 1A-1, TPEN completely removed the OGD-induced Zn\(^{2+}\) increases, including the first and second rises. This result not only supported that OGD-induced Zn\(^{2+}\) rises detected with fluorescent Zn\(^{2+}\) indicator was a Zn\(^{2+}\) dependent phenomenon, but also indicated that they were originated from intracellular Zn\(^{2+}\) storage. Zn\(^{2+}\) is found in cellular organelles such as endoplasmic reticulum (ER) (14, 42, 59, 70, 72), with ER requirements of Zn\(^{2+}\) for its normal function (21, 22).

Thapsigargin (TG) is an inhibitor of the sarco/ER Ca\(^{2+}\) ATPase (SERCA) and is widely used for Ca\(^{2+}\) store depletion by pre-incubation with low concentration of TG. We showed previously that TG also increased [Zn\(^{2+}\)], by releasing Zn\(^{2+}\) from TG-sensitive and IP3R-mediated stores of the ER (42, 70). In this study, we incubated cells with TG (2 µM) for 20 minutes prior to inducting OGD conditions. Since TG was dissolved in DMSO, cells exposed to DMSO (0.1%) were used as a control for this set of experiments. Control cells responded to OGD treatment with smaller Zn\(^{2+}\) rises (Figure 2A), however we still observed two Zn\(^{2+}\) rises with overall response resembling OGD-induced Zn\(^{2+}\) changes seen in Figure
1. In comparison, the cells that were treated with TG showed little Zn\(^{2+}\) increases in response to hypoxia treatment (Figure 2A). Baseline of thapsigargin treated cells is normalized to elevated Zn\(^{2+}\) level, prior to the induction of OGD. These results showed that TG had a significant influence on the OGD-induced Zn\(^{2+}\) response in HeLa cells, which suggests that ER is as a potential major source of free Zn\(^{2+}\) and that ER can attribute to Zn\(^{2+}\) dyshomeostasis. Thus, the ability of both TPEN and TG to limit OGD-induced Zn\(^{2+}\) rises supports that the observed Zn\(^{2+}\) during chemical ischemia is of an intracellular origin and is derived from intracellular stores.

To determine if mitochondrial Zn\(^{2+}\) pool was contributing to OGD-induced Zn\(^{2+}\) increases, we mobilized mitochondrial Zn\(^{2+}\) by the application of FCCP, a mitochondrial uncoupler that dissipates the proton gradient across the inner mitochondrial membrane. Prior studies have found that FCCP releases Zn\(^{2+}\) from mitochondria and also disrupts the uptake of Zn\(^{2+}\) into the mitochondria (50, 62). In this study we observed a significant FCCP-induced [Zn\(^{2+}\)], rises (measured with FluoZin-3, AM) when cells were bathed in a physiological buffer with FCCP (1µM) (Figure 2B). These results supports that Zn\(^{2+}\) can be released into the cytosol by FCCP. Next, to learn if the release of Zn\(^{2+}\) from mitochondria by impairing mitochondrial functions was responsible for OGD-induced Zn\(^{2+}\) rises, we pre-incubated cells with FCCP (prior to OGD treatment), these experiments are independent form the above described experiments and were done separately. Cells were pre-treated with FCCP (1µM) for 10 minutes, then FCCP was washed out before start of OGD treatment. The data from FCCP pretreated cells were then compared to the data from cells without FCCP pretreatment, where the FCCP treated cells were normalized to increased Zn\(^{2+}\) during baseline. Under this condition, we still observed the increases in [Zn\(^{2+}\)], with two sharp rises. The first Zn\(^{2+}\) transient was not affected by FCCP pre-treatment and remained generally unchanged in onset, duration and amplitude. The second OGD-induced Zn\(^{2+}\) rise, however, was significantly reduced compared to the cells without FCCP pretreatment (Figure 2C), indicating that the release of mitochondrial Zn\(^{2+}\) may be a major contributor to the second rise of [Zn\(^{2+}\)]. To quantify the change in the second Zn\(^{2+}\) rise, the fluorescent intensity of the second rise was divided by the intensity of the first rise of [Zn\(^{2+}\)], in the same cell and the data is presented as a ratio (Figure 2D). Overall, the second OGD-induced [Zn\(^{2+}\)], rise is greater than the first OGD-induced rise with ratio of \(2^{\text{nd}}/1^{\text{st}} > 1\). In the cells pretreated with FCCP the second Zn\(^{2+}\) rise was less than half the amplitude of the first Zn\(^{2+}\) rise with ratio \(2^{\text{nd}}/1^{\text{st}} < 0.5\).

\(\text{Zn}^{2+}\) mediates mitochondrial ROS accumulation - We previously showed that Zn\(^{2+}\) fluorescence was co-localized in mitochondria when it was labeled with mitochondrial membrane indicator MitoFluor Red (42) and MitoTracker Red (52). It is consistent with the notion that mitochondria are Zn\(^{2+}\) storing organelles containing free Zn\(^{2+}\) (62, 65). Figure 3A shows the co-localization of Zn\(^{2+}\) and mitochondrial ROS fluorescence. For this set of experiments, to show the mitochondrial origin of ROS production, mitochondria were labeled with MitoSOX Red (5µM), a ROS indicator sensitive to mitochondrial superoxide production, where oxidation of MitoSOX Red by mitochondrial superoxide produces red fluorescence. Mitochondrial Zn\(^{2+}\) fluorescence was detected with Zinpyr-1 which is lipophilic and easily penetrates plasma and mitochondrial membranes, which yielded relative strong signal of Zn\(^{2+}\) fluorescence. We observed a strong co-localization of two fluorescent signals in merged images (Figure 3A) that resemble previous co-localization study of Zn\(^{2+}\) and mitochondria (42). In addition, the co-localization was also analyzed using NIS-element (Nikon) co-localization software, where we carefully outlined multiple selected mitochondria and calculated co-localization with Mander’s overlap coefficient of 0.95 ± 0.02 and Pearson’s correlation coefficient of 0.85 ± 0.05. Taking into account the visual inspection of merged images (Figure 3A) and coefficients of co-localization (both Pearson’s and Mander’s), we concluded that mitochondria labeled with Zn\(^{2+}\) fluorescence were also undergoing mitochondrial ROS productions.

Next, we wanted to determine if mitochondrial ROS production were sensitive to Zn\(^{2+}\) accumulation. We used MitoSOX Red as a mitochondrial ROS fluorescent probe. The control cells were exposed to physiological buffer, under normoxic conditions. When cells were treated with OGD buffer, the MitoSOX Red fluorescence increased significantly within 5 minutes after started OGD indicating the...
rising of \([\text{ROS}]_{\text{mito}}\) production. There were generally two \([\text{ROS}]_{\text{mito}}\) rises during 30 minutes of OGD treatment (Figure 3B; also see Figure 1C). When we removed intracellular \(\text{Zn}^{2+}\) with TPEN during OGD, we observed only one mitochondrial ROS rise. Overall, in the presence of TPEN, OGD-induced mitochondrial ROS rise was delayed almost 10 minutes and was significantly smaller in amplitude. These data suggest that \([\text{ROS}]_{\text{mito}}\) production under OGD conditions is sensitive to \(\text{Zn}^{2+}\) accumulation. In another set of experiments, FCCP application (1 \(\mu\)M) induced a steady increase of ROS in cells labeled with MitoSOX Red (Figure 3C). In the presence of TPEN, FCCP induced a significantly smaller ROS accumulation, supporting further that \(\text{Zn}^{2+}\) was an important factor in sustained mitochondrial ROS production.

**NADPH oxidase contributes to \([\text{ROS}]_{\text{mito}}\) accumulation induced by \(\text{Zn}^{2+}\)**: In following study, we examined the generation of ROS in response to the application of exogenous \(\text{Zn}^{2+}\) with a purpose to identify the possible mechanism in which the accumulation of \([\text{Zn}^{2+}]\) leads to ROS generation. HeLa cells were labeled with MitoSOX Red and exposed to exogenous \(\text{Zn}^{2+}\) (50 \(\mu\)M) along with a \(\text{Zn}^{2+}\) ionophore sodium pyrithione that transports \(\text{Zn}^{2+}\) across a cell membrane and increases intracellular \(\text{Zn}^{2+}\) concentration. In reality by adding the zinc/ionophore combination, we are studying a rapid intracellular \(\text{Zn}^{2+}\) increase during normoxia, or in other words – \(\text{Zn}^{2+}\) induced changes. As a contrast, the control cells were bathed in physiological buffer. All of these experiments were done under normoxic conditions. \(\text{Zn}^{2+}\) addition caused significant increases in \([\text{ROS}]_{\text{mito}}\) production which started 10-15 minutes after addition of \(\text{Zn}^{2+}\). Further in the paper we will be calling this a \(\text{Zn}^{2+}\)-induced \([\text{ROS}]_{\text{mito}}\), to differentiate these data from OGD-induced ROS. Next, we examined \(\text{Zn}^{2+}\)-induced ROS generation in the presence of oxipyrinol, a xanthine oxidase inhibitor, or apocynin, a NADPH oxidase inhibitor (Petronio 2013). Both enzymes have been shown to be significant contributors to ROS generation. As show in Figure 4A, the inhibition of xanthine oxidase by oxipyrinol (60 \(\mu\)M) did not affect \(\text{Zn}^{2+}\)-induced ROS accumulation. On the other hand, \(\text{Zn}^{2+}\)-induced ROS production was significantly reduced when NADPH oxidase was inhibited by apocynin (60 \(\mu\)M) (Figure 4A), suggesting that \(\text{Zn}^{2+}\)-induced ROS accumulation involves the activation of NADPH oxidase.

To further study if NADPH oxidase mediated \(\text{Zn}^{2+}\)-induced ROS accumulation, we examined whether the application of \(\text{Zn}^{2+}\) will alter the amount of the functional component of NADPH oxidase - p47phox (NCF1) enhances the functional activation of NADPH oxidase in HeLa cells. NADPH oxidase subunit p47phox has been used as a proxy of NADPH oxidase amount (8, 37). In this experiment, HeLa cells were exposed to following treatments: physiological buffer, pyrithione alone, and pyrithione plus \(\text{Zn}^{2+}\). Representative western blot is shown in Figure 4B. The antibody used detects endogenous total p47phox and as expected, we did not detect a lot of p47phox in physiological or pyrithione alone conditions, however there were significantly increased levels of the NADPH oxidase subunit p47phox in \(\text{Zn}^{2+}\)-treated cells. Interestingly, these detectable changes were observed within 30 minutes of treatment, which is very short time for protein induction, suggesting that \(\text{Zn}^{2+}\) action on NADPH oxidase is very fast.

Both \(\text{Zn}^{2+}\) and NADPH oxidase contribute to ROS accumulation during re-oxygenation - The accumulation of ROS during re-oxygenation has been well described as a possible contributor to reperfusion or re-oxygenation injury (4). There are evidence that NADPH oxidase contributes significantly to ROS generation following re-oxygenation or reperfusion (1, 9, 30). Here, we examined the effect of apocynin, an NADPH oxidase inhibitor, on ROS generation during re-oxygenation, which was compared to the effect of \(\text{Zn}^{2+}\) removal by TPEN on the ROS production. The experiments were carried out in freshly prepared rat hippocampal brain slices. The advantage of the hippocampal brain slices is that it is an established *in situ* model for neural function and injury, and is particularly sensitive to ischemia (OGD) and re-oxygenation. Ischemia like condition was achieved by perfusing brain slices with oxygen and glucose deprived (OGD) physiological buffer (artificial cerebral spinal fluid, ACSF), subsequently, slices were reperfused with normal ACSF to achieve re-oxygenation. The slices were
loaded with a ROS indicator dihydroethidium (HEt) (see detail in Materials and Methods). Apocynin (180 µM) or TPEN (35 µM) were administrated in the perfusate during re-oxygenation. As summarized in Figure 4C, re-oxygenation caused a significant increase in HEt fluorescence compared to HEt fluorescence with OGD. Both treatments with apocynin and TPEN significantly reduced the generation of cellular ROS during re-oxygenation. The inhibition of ROS production by TPEN was significantly greater than that by apocynin (Figure 4C). Taken together, we have demonstrated that NADPH oxidase mediates Zn\textsuperscript{2+}-induced ROS accumulation (Figure 4A) and that Zn\textsuperscript{2+} enhances the amount of functional subunit p47phox of NADPH oxidase (Figure 4B). The results indicate that both Zn\textsuperscript{2+} and NADPH oxidase are contributing to ROS accumulation during re-oxygenation with Zn\textsuperscript{2+} accumulation possibly precipitating ROS accumulation by activating NADPH oxidase.

**DISCUSSION**

*Two distinct Zn\textsuperscript{2+} rises in multi-phase responses during OGD*- In the present study, we applied sodium dithionite to live cells, which rapidly binds oxygen in solution, to induce consistent hypoxic like state in cells, as many other groups have done (23, 29, 41, 43, 56, 60, 77). We observed changes in cytosolic free Zn\textsuperscript{2+} that followed a consistent pattern that could be divided into four phases of two distinct Zn\textsuperscript{2+} rises (Figure 1). The first phase (I) is a rapid rising of Zn\textsuperscript{2+} transient elicited soon after induction of OGD condition. Zn\textsuperscript{2+} then recovered toward basal levels during the second phase (II) as a descending phase of Zn\textsuperscript{2+} transient, indicating that increased Zn\textsuperscript{2+} was not necessary to sustain homeostasis changes if OGD was brief. Therefore, the first Zn\textsuperscript{2+} rise is a transient increase of cytosolic free Zn\textsuperscript{2+}. It was observed in all recorded cells, suggesting a uniform response to OGD and the response was consistent in the onset of phase I. However, there was a difference in phase II observed among cells, where the descending part of the Zn\textsuperscript{2+} transient came back toward the baseline. In most cells the Zn\textsuperscript{2+} level reduced but stayed elevated. Since cells were continuously under the OGD in this study, the Zn\textsuperscript{2+} rise didn’t completely return to basal levels in the most cells. There was a latent phase (III) between first and second Zn\textsuperscript{2+} rises, during which intracellular free Zn\textsuperscript{2+} level maintained at above the basal level. The fourth phase (IV) was marked by a quick increase in the intracellular free Zn\textsuperscript{2+} to a sustained plateau in the significantly higher amplitude than the first phase from which Zn\textsuperscript{2+} increases didn’t recover. We have termed this delayed Zn\textsuperscript{2+} elevation as Zn\textsuperscript{2+} overload.

The principal finding of this study is the occurrence of early phases (phases I&II) and latent phase (phase III) of Zn\textsuperscript{2+} response following OGD. Early Zn\textsuperscript{2+} transient increase is adaptive and protective responses to hypoxic treatment. Studies suggest that the increased Zn\textsuperscript{2+} concentrations had a pro-antioxidant effect by Zn\textsuperscript{2+} binding to thiols and preventing their oxidation, and/or by activating antioxidant response elements (46, 57). Many radical scavengers such as Cu–Zn-SOD are Zn\textsuperscript{2+} containing proteins, therefore, the moderate increase in cytosolic Zn\textsuperscript{2+} may be protective to cells. The key feature of the phase II is that the Zn\textsuperscript{2+} transient appear to descend toward the basal levels in all cells, which indicates that a short hypoxic treatment does not irreversibly changes Zn\textsuperscript{2+} homeostasis and suggesting that Zn\textsuperscript{2+} buffering and homeostatic mechanisms were intact. Since maintaining basal Zn\textsuperscript{2+} requires the concerted efforts of a number of regulatory and metabolic processes, the finding that this system is functional following OGD suggests that the OGD induced cellular process that may be reversible at this time during a short exposure to OGD. As OGD continues, we observe latent phase (III), during which, Zn\textsuperscript{2+} remains elevated (Figure 1). At this point the Zn\textsuperscript{2+} buffering systems may become overwhelmed, and in addition, the increased Zn\textsuperscript{2+} may trigger other adverse cellular responses, like ROS production (40, 44), which in turn may further disturb the buffering systems, causing total loss of Zn\textsuperscript{2+} homeostasis, which is mark by Zn\textsuperscript{2+} overload (phase IV). This second significant zinc increase, was almost double the amplitude of the first increase and rapidly reached a plateau, suggesting irreversible dysfunction of Zn\textsuperscript{2+} homeostasis (3, 13).
Sources of intracellular Zn\(^{2+}\) contributing to Zn\(^{2+}\) transient - The presence of such a consistent and large Zn\(^{2+}\) response to OGD was in itself a fascinating observation, however we wanted to explain a source for these two Zn\(^{2+}\) responses (Figure 1&2). When TPEN was applied, before cells were exposed to OGD, both the first and the second rise were abolished (Figure 1A.1). During the hypoxic experiments the extracellular solutions did not have detectable amount of Zn\(^{2+}\), suggesting that the both rises we observed during OGD without TPEN, are of intracellular origin, which is not surprising due to mounting evidence that Zn\(^{2+}\) is bound to proteins and stored in cellular organelles (44).

Emerging evidence has shown that Zn\(^{2+}\) transporters are found on plasma membrane of cellular organelles providing molecular evidence that Zn\(^{2+}\) is transported among discrete subcellular compartments (34, 40). Zn\(^{2+}\) can accumulate in the mitochondrial matrix (See below). ER has been emerging as a site of Zn\(^{2+}\) storage (14, 42, 59, 70, 72), where Zn\(^{2+}\) is required for normal ER function (21, 22). Worthy of notice is that ER and mitochondria are key organelles involved in the storage and release of intracellular Ca\(^{2+}\) (but also see (47, 69)). In the present study, the timescale of Zn\(^{2+}\) transient observed soon after starting hypoxic treatment is similar to Ca\(^{2+}\) waves that is attributed to the release and uptake of Ca\(^{2+}\) by the ER. We previously showed that this ER Zn\(^{2+}\) pool can be readily mobilized when cells are treated with TG (70). Consistent with previous study, the present study found that TG pretreatment abolishes Zn\(^{2+}\) rises in cells undergoing OGD (Figure 2A). Thus, the ability of both TPEN and TG to limit OGD-induced Zn\(^{2+}\) elevations supports the hypothesis that the observed Zn\(^{2+}\) increases are of intracellular origin and are derived from intracellular stores.

An interesting question is how the elevated Zn\(^{2+}\) is so quickly buffered as seen in phase II or the descending phase of the first Zn\(^{2+}\) transient. The considerably quick response observed in hypoxic treatment also suggests a fast kinetics of Zn\(^{2+}\) mobilization and removal. We suggest that intracellular Zn\(^{2+}\) storages, such as MTs, ER and mitochondria, can play dual role as both a Zn\(^{2+}\) source and a Zn\(^{2+}\) sink. For example, mammalian Zn\(^{2+}\) transporter ZIP7 proteins are present in the ER, where they contribute to the release of free Zn\(^{2+}\) into the cytosol from ER stores (32, 72). On the other hand, Zn\(^{2+}\) transporters ZnT5/ZnT6/ZnT7 are involved in ER homeostasis by transporting Zn\(^{2+}\) into the lumen (33, 71), and may also function as bidirectional transporters (20, 21, 58). Furthermore, the elevated level of Zn\(^{2+}\) causes the biological system to store the extra Zn\(^{2+}\) ions into the mitochondrial matrix, another crucial player in Zn\(^{2+}\) clearance (see below). The prolonged OGD exposure causes the continuous release of Zn\(^{2+}\) from above discussed stores, which may contribute to Zn\(^{2+}\) and ROS accumulation seen in the latent phase (phase III).

Mitochondrial dyshomeostasis and MTs are a major sources of the second Zn\(^{2+}\) increase - There has been considerable focus on mitochondria taking up cytosolic Zn\(^{2+}\) to maintain intracellular Zn\(^{2+}\) concentration (62). We previously reported that both Zn\(^{2+}\) and mitochondria co-localize in healthy live cells (42). Other groups showed that application of FCCP mobilized mitochondrial Zn\(^{2+}\) pool (50, 63). In the present study we observed substantial increase in cytosolic Zn\(^{2+}\) after 2 minutes of treatment with FCCP (Figure 2B), which supports that mitochondria may serve as a source of free Zn\(^{2+}\). Further tests indicate that mitochondrial dyshomeostasis acts as a source of second Zn\(^{2+}\) increase or Zn\(^{2+}\) overload (Figure 2C&D). Taking into account the visual inspection of merged images of cells double stained with Zn\(^{2+}\) and mitochondrial ROS dyes, our study supports the notion that mitochondria is Zn\(^{2+}\) storing organelle and is also undergoing mitochondrial ROS production during stress under OGD (62, 65). Studies have shown that Zn\(^{2+}\) transporters, or the mitochondrial calcium uniporter, are involved in Zn\(^{2+}\) uptake by mitochondria (20, 51). The mitochondrial Zn\(^{2+}\) uptake and membrane depolarization is also associated with Zn\(^{2+}\) accumulation following OGD (65) (see Figure 5C). Thus, Zn\(^{2+}\) uptake by mitochondria may be responsible for phase II or the descending phase of early Zn\(^{2+}\) transient (Figures 1&5). While the Zn\(^{2+}\) uptake may provide clearance of cytosolic Zn\(^{2+}\) in cells under OGD, continuous and consistent Zn\(^{2+}\) accumulation in mitochondrial lumen alter or, consequently, impair mitochondrial function, leading to the opening of the mPTP (7, 50). A consequence of mPTP opening is the efflux of Zn\(^{2+}\) from mitochondria (50) which contributes to Zn\(^{2+}\) elevation in the phase III and is a causal factor of...
massive \( \text{Zn}^{2+} \) overload seen in the phase IV. Hence, there is a biphasic control of cytosolic \( \text{Zn}^{2+} \) by mitochondria in response to the rising \( \text{Zn}^{2+} \): early uptake, to remove cytosolic \( \text{Zn}^{2+} \) and late release, due to mitochondrial dysfunction, which causes \( \text{Zn}^{2+} \) overload. This provides novel basis for complex pathological patterns of intracellular \( \text{Zn}^{2+} \) signaling. This study shows that TPEN application delayed the OGD-induced mitochondrial ROS accumulation as well as reduced FCCP-induced mitochondrial ROS production (Figure 3B& C), supporting the notion that the mitochondrial \( \text{Zn}^{2+} \) uptake and accumulation may lead to a loss of mitochondrial membrane potential and a subsequent increase in ROS production (50, 51).

We hypothesize that another critical source of this \( \text{Zn}^{2+} \) may be the abundant metallothioneins (MTs) that bind as much as 5-10% of all cellular \( \text{Zn}^{2+} \), from which \( \text{Zn}^{2+} \) could be released rapidly by OGD-induced ROS (45). The sequestration and storage of \( \text{Zn}^{2+} \) in MTs have been extensively investigated. MT have been shown to be the sink as well as a source of \( \text{Zn}^{2+} \), and they help to maintain \( \text{Zn}^{2+} \) homeostasis (15). In OGD, there are abnormal cellular conditions such as acidosis, nitrosylation, lipid peroxidation products, and glutathione disulfide, all of which favor \( \text{Zn}^{2+} \) dissociation from MTs, resulting in increases in intracellular free \( \text{Zn}^{2+} \) (26, 40, 49). For example, prior studies have found that \( \text{Zn}^{2+} \) binding to MTs is decreased at acidic pH (36). \( \text{Zn}^{2+} \) dissociation from MTs may contribute to the \( \text{Zn}^{2+} \) overload (phase IV) of \( \text{Zn}^{2+} \) transient seen in the prolonged OGD. These data will need to be confirmed with inhibitors specific to MTs.

Mechanisms of \( \text{Zn}^{2+} \)-induced ROS accumulation - OGD-induced \( \text{Zn}^{2+} \) rises were not the only focus of this study. Our results indicate that \( \text{Zn}^{2+} \) is a causal factor of OGD-induced ROS accumulation. We examine the temporal relationship between the two important phenomena, where early \( \text{Zn}^{2+} \) transient (phase I and II) preceded the early ROS accumulation. Interestingly, the second \( \text{Zn}^{2+} \) rise (phase IV) appears also to precede second ROS accumulation. As discussed above, mitochondria can shape and maintain \( \text{Zn}^{2+} \) homeostasis by actively taking up \( \text{Zn}^{2+} \) into mitochondria matrix, which is a protective mechanism to curtail \( \text{Zn}^{2+} \) imbalance caused by a short OGD. However, an excess of \( \text{Zn}^{2+} \) in the mitochondrial matrix may disrupt most of the enzymes of the oxidative phosphorylation, and leads to the oxidative stress and membrane impairment. A consequence of excessive \( \text{Zn}^{2+} \) accumulation is the increased generation of ROS (7, 50). We found that \( \text{Zn}^{2+} \) facilitates ROS production based on multiple lines of evidence. The application of \( \text{Zn}^{2+} \)-induced the steady ROS production (Figure 4A). TPEN significantly delayed OGD-induced and FCCP-induced ROS increases (Figure 3B& C). It is important to note that TPEN didn’t completely remove ROS increases due to possibly \( \text{Zn}^{2+} \)-independent ROS production within mitochondria. Taking all the above data into account, we suggest that \( \text{Zn}^{2+} \) is required for the induction of mitochondrial ROS accumulation and precedes its onset. To our knowledge we are the first to report this kind of temporal crosstalk between \( \text{Zn}^{2+} \) and mitochondrial ROS.

OGD-induced ROS production is a very complicated cellular response with the mechanism and key factors that are still unclear. By applying exogenous \( \text{Zn}^{2+} \) to cells under normoxic condition and observing the mitochondrial ROS, we collected valuable data because we were only changing \( \text{Zn}^{2+} \) concentration and these data are more straightforward to interpret. The application of \( \text{Zn}^{2+} \) induced a large mitochondrial ROS increase (Figure 4A), which was consistent with results that \( \text{Zn}^{2+} \) removal by TPEN delayed or reduced mitochondrial ROS production induced by OGD or FCCP (Figure 3B& C), as discussed above. Besides mitochondria, \( \text{Zn}^{2+} \) also appears to interact with NADPH oxidase, because \( \text{Zn}^{2+} \)-induced ROS accumulation was significantly reduced with inhibition of NADPH oxidase (Figure 4A). To further solidify these findings, we used western blot technique to quantify the amount of p47phox, which is one of the functionally important subunits of NADPH oxidase in HeLa cells that were exposed to present in cells treated with exogenous \( \text{Zn}^{2+} \) treatment with addition of \( \text{Zn}^{2+} \) ionophore, which when combined, translates to intracellular \( \text{Zn}^{2+} \) increase. Intracellular \( \text{Zn}^{2+} \) increase induced higher levels of p47phox, which creates possibility for increase in NADPH oxidase (Nox) in the cells. The increase happened in very short period of time (30 minutes), while compared to controls cells had very low expression in both physiological control as well as ionophore alone control (Figure 4B). p47phox has
been shown to be important component of Nox1 (3, 73), Nox2 (5) and Nox3 (73); however, a recent comprehensive review, suggests that Nox2 is clearly associated with p47phox (55). In light of previous reports of zinc and NADPH oxidase interaction (38, 48, 53) and taking into account data reported above, we suggest that NADPH oxidase is a major contributor to Zn$^{2+}$-induced ROS in HeLa cells and that the Zn$^{2+}$ induces ROS through the upregulation of p47phox, a functional subunit of NADPH oxidase (Nox2).

Recently, there is emerging evidence that NADPH oxidase, contributes significantly to ROS generation following reperfusion in neurons (1, 9, 30). However, the molecular mechanism for so-called ‘burst’ of ROS generation remains largely uncertain. In this study we report that both NADPH oxidase inhibitor and Zn$^{2+}$ removal by TPEN significantly inhibited reoxygeration-induced ROS increases (Figure 4C), which further solidifies the importance of Zn$^{2+}$ and NADPH oxidase interaction. In summary, we present here for the first time, that there are multiple intracellular Zn$^{2+}$ rises during hypoxic exposure, revealing rather interesting and complicated crosstalk between ROS and Zn$^{2+}$ which involves multiple factors. As depicted in Figure 5, OGD triggers Zn$^{2+}$ release from the ER stores. It is possible that the early Zn$^{2+}$ transient may be protective in response to short OGD, but, at the same time, it makes cells vulnerable if OGD continues. Zn$^{2+}$ surge was quickly buffered by normal cellular sequestration processes, and Zn$^{2+}$ may return to basal level if OGD cease. Specifically, Zn$^{2+}$ is sequestered in mitochondria through the activation of a cation-permeable channel or other unidentified - independent pathway (16, 27, 28, 35, 50). Excessive and prolonged intra-mitochondrial Zn$^{2+}$ overload inhibits the activity of complex III of the electron transport chain, or by interfering with complex I and α-ketoglutarate dehydrogenase (KGDHG) (62), and the activation of the mitochondrial permeability transition pore (MPTP) (6, 7, 13, 28, 64). Therefore, mitochondria may become the first victim of prolonged Zn$^{2+}$ increase. In return, injured mitochondria release ROS and Zn$^{2+}$, which worsen dyshomeostasis and trigger the pro-apoptotic signaling cascades pathways. Extensive increase in [Zn$^{2+}$], may be a causal factor of ‘burst’ ROS generation seen in oxidation as Zn$^{2+}$ interacts with NADPH oxidase and causes further ROS increase and Zn$^{2+}$ overload.

**EXPERIMENTAL PROCEDURES**

**Materials and reagents** – Most chemicals were purchased from Sigma-Aldrich (St. Louis, MO), with exception of apocynin which was purchased from Santa Cruz Biotechnologies (Dallas, TX). Fluorescent dyes were purchased from: MitoSOX Red dye (Molecular Probes, Eugene, OR) and FluoZin-3, AM (Life Technologies, Grand Island, NY). HeLa cells were purchased from ATCC (Manass, VA). Western blot materials and reagents were purchased from Bio-Rad and Invitrogen. Antibodies were from Cell Signaling.

**Cell culture** – HeLa cells were used between passages 4-14. They were split every other day using the standard trypsanization method and maintained in EMEM medium supplemented with 5% fetal bovine serum (ATCC, Manass, VA) in 5% CO$_2$ -95% humidity air at 37°C (as suggested by ATCC).

**Fluorescent experiments** - HeLa cells were trypsinized and seeded at medium density onto glass bottom Petri dishes (P35G-4.5-14-C; MatTek Corp, Ashland, MA). Cells were incubated in 5% CO$_2$ - 95% humidity air at 37°C for at least 24 hours before experimentation. On the day of the experiment, the cells were washed three times with 1 mL of physiological buffer (in mM): 25 HEPES, 125 NaCl, 3 KCl, 1.28 CaCl$_2$, 1.1 MgCl$_2$, 5 glucose, pH 7.4. For mitochondrial superoxide detection, MitoSOX Red dye was used at final concentration of 5µM and cells were loaded for 10 min at 37°C. For Zn$^{2+}$ detection, FluoZin-3, AM was used at final concentration of 1µM and loaded onto cells for 60 minutes at room temperature. After incubation with respective dyes for each treatment, cells were washed three times with physiological buffer and left to “rest” at room temperature for 10 minutes before experimentation. Images were collected with a Motic AE31 microscope using Olympus U Plan FL 40X, 075 NA, with QImaging Retiga 1300i camera, with 40X/0.75 Olympus objective. Image-Pro Plus 6.2 (Media Cybernetics, Rockville, MD) was used to collect and analyze the data.
Chemical Ischemia and OGD - Chemical ischemia was induced with hypoxic buffer by using 4 mM final concentration of sodium dithionite (DT) in oxygen and glucose deprived (OGD) physiological buffer (66). To achieve OGD, nitrogen gas was bubbled through the physiological buffer for at least 10 minutes before the experiment. This OGD and 4 mM sodium dithionite buffer was added by pipetting it as a 2x concentrated solution into the petri dish holding the cells, to induce rapid and reliable hypoxia like condition (23, 43, 77), after at least one minute of the baseline fluorescence was observed. The recordings lasted for 30 minutes.

Co-localization experiments – HeLa cells were simultaneously preloaded with mitochondrial ROS indicator MitoSOX Red (5μM) and free Zn^{2+} indicator Zinpyr-1 (5μM) at 37°C for 10 minutes, then followed the same protocol as described above. Confocal system Nikon A1R was used with Nikon Eclipse Ti microscope, and Nikon NIS- Elements, version 4 software. Zinpyr-1(11, 74) (K_D = 0.7 ± 0.1 nM) was chosen for this experiment because this sensor is lipophilic and easily penetrates plasma and mitochondrial membranes, which yielded relative strong signal of Zn^{2+} fluorescence. The co-localization was also analyzed by using ROI and NIS-element (Nikon) co-localization software, where we carefully outlined multiple randomly selected mitochondria and calculated Pearson’s correlation coefficient and Mander’s overlap coefficient of the same mitochondria, using the software. We used Dunn’s suggestions in interpreting the co-localization data (19).

NADPH oxidase and xanthine oxidase inhibition – HeLa cells were prepared using the same protocols as above (fluorescent experiments). Cells were loaded with MitoSOX Red (5μM). The NADPH oxidase inhibitor apocynin was dissolved in DMSO and used at 60 μM final concentration with exogenous Zn^{2+} (50μM) and sodium pyrithione (10μM). Apocynin was added at the same time as the other solutions. All the solutions had 0.1% final concentration of DMSO, as a control. Xanthine oxidase was inhibited by 60 μM oxypurinol, experiments were performed as for NADPH oxidase inhibition. Fluorescent intensity was measured before the addition of treatment solutions and was measured again after 30 minutes of treatment.

Immunoblot (Western Blot) – HeLa cells were treated and plated as described in fluorescent experiments. For these experiments 35mm sterile culture dishes were used, instead of glass bottom dishes. After 24 hours of culture, the HeLa cells were washed three times with physiological buffer and solutions with appropriate treatments were added to the cells. Cells were incubated for 30 minutes in 37°C incubator. After the incubation, the experimental solutions were washed out once with physiological buffer and removed from the samples. 1.5X Laemlli sample buffer (Bio-Rad) was added at 100 μL per each 35 mm culture dish. Cells were scraped off and homogenized in the sample buffer. Samples were boiled for 5 minutes. Proteins were separated immediately by SDS-PAGE electrophoresis on 10% acrylamide/bis gel with XCell II module (Bio-Rad). PVDF 0.45μm membrane (Invitrogen) was used to transfer the proteins using XCell II module at 300 mA constant voltage for 2 hours at room temperature. Membrane was blocked with 5% BSA in TBS with 5% TritonX (TBST) for 2 hours at room temperature. Rabbit monoclonal antibody against p47phox was diluted 1:1000 in 2%BSA/TBST and membrane was incubated with the primary antibody solution overnight at 4°C. The p47phox antibody was purchased from cell signaling (#4301, lot # 1) and is specific to total exogenous p47phox (NCF1). Horse radish peroxidase (HRP) labeled secondary antibody anti- rabbit was used (cell signaling #7074). ChemiDoc system (Bio-Rad) was used to visualize the signal with Clarity Western ECL Substrate (Bio-Rad), this reagent was used as specified by manufacturer. Total protein staining on the same blot was used as a loading control. To stain total proteins on the blot, coomassie brilliant blue R-250 (Bio-Rad) was used, the membrane was incubated for 1 minute and then stain was washed off three times, protocol described in details in (76). The advantages of using total protein over the traditional housekeeping proteins is well described in (76). The washed membrane was dried up completely and imaged with ChemiDoc imaging system with colorimetric blot setting.

Brain slices - Brain slices were prepared from male Sprague Dawley rats from 4 to 6 month old, raised in normal laboratory conditions with standard rat food. Animal protocol was approved by Institutional Animal Care and Use Committee (IACUC). Hippocampus was dissected out of the brain and...
sliced to 200µm thickness and were incubated immediately with artificial cerebral spinal fluid (ACSF) (in mM): 121 NaCl; 1.75 KCl; 1.3 MgCl\(_2\); 2.5 CaCl\(_2\); 1.25 KH\(_2\)PO\(_4\); 26 NaHCO\(_3\) 10 glucose, gassed with 95\%O2/5\%CO2, pH 7.2-7.3, for 1 hour to recover from slicing in interface chamber. After recovery, slices were transferred to a dye incubation chamber, containing 2ml of ACSF and bubbled with 95\%O2/5\%CO2 and loaded with 20µM HEt dye for 30 minutes. After loading the dye was removed and slices were washed three times with ACSF prior to the experiments. For oxygen-glucose deprivation (OGD), all slice manipulations were performed in 35 mm glass-bottomed dishes, where slices were fully submerged in circulated oxygen-glucose-deprived ACSF solution, which was pre-bubbled with 95\%N2/5\%CO2 gas for 30 minutes before slice immersion, to ensure oxygen removal. Baseline fluorescence was measured for 5-10 minutes prior to exposure to OGD. OGD exposure was implemented for 30 minutes, where slices were perfused continually. This treatment was followed by subsequent reperfusion with normal ACSF for 40 minutes (re-oxygenation or re-perfusion). The fluorescence was recorded before re-oxygenation and 30 minutes after re-oxygenation. The regions of interest (ROIs) in CA1 region of hippocampus were highlighted and fluorescence was quantifies using Image Pro software, as in above described fluorescent experiments. Apocynin, NADPH oxidase inhibitor (180µM) or TPEN, Zn\(^{2+}\) specific chelator (35µM) were added to perfusion during hypoxia and re-oxygenation only, and were not present during hypoxia. The fluorescent measurements were compared to the brain slices without any treatment during hypoxia and re-oxygenation.

**Data analyses** – For Zn\(^{2+}\) and mitochondrial ROS transients, the cytosol excluding the nucleus was analyzed for each cell. The changes in fluorescence (Δ F) in the cytosol were background corrected by subtracting fluorescence from area without cells. Each relative fluorescence trace was normalized to baseline by the following formula \(\Delta F = (F_{\text{measure}} - F_0)/F_0\), where \(F_{\text{measure}}\) is a recorded data point, \(F_0\) is relative fluorescence at baseline. The averages of the traces were plotted on the graph with standard deviation bars or a representative trace was selected and plotted. For Zn\(^{2+}\)-induced ROS experiments and enzyme inhibitors, the percent change in fluorescence was calculated following formula: \((F - F_0)/F_0\) x 100, where \(F_0\) is averaged baseline fluorescence intensity before addition of experimental solutions and F is fluorescence intensity value after 30 minutes of incubation with the experimental solutions. The significance was measured by simple two-group comparison and analyzed by Student’s paired t test (or single-factor ANOVA), with \(p < 0.05\) considered significant.

**FOOTNOTES**

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The abbreviations used are: ROS, reactive oxygen species; TPEN, N,N,N',N'-tetrakis-(2-pyridylmethyl)ethylenediamine; DT, sodium dithionite; OGD, oxygen glucose deprivation; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMSO, dimethyl sulfoxide; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; XO, xanthine oxidase; NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase; p47phox, neutrophil cytosolic factor 1; BSA, bovine serum albumin; TBS, tris(hydroxymethyl)aminomethane buffered saline; HRP, horse radish peroxidase; ACSF, artificial cerebral spinal fluid; ROIs, regions of interest; HEt, hydroethidine or dihydroethidium; PVDF membrane, polyvinylidene difluoride membrane.

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**Conflict of interest:** The authors declare that they have no conflict of interest with the content of this article.
Crosstalk between intracellular zinc and ROS in chemical ischemia

**Author contributions:** KGS researched data, contributed to discussion, wrote, reviewed and edited the manuscript. QL collected brain slice data, contributed to discussion and edited the manuscript. YVL conceived and coordinated the study, researched data, contributed to discussion and edited the manuscript and references. All authors approved the final version of the manuscript.

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Crosstalk between intracellular zinc and ROS in chemical ischemia


Crosstalk between intracellular zinc and ROS in chemical ischemia


**FIGURE LEGENDS**

**FIGURE 1.** Zinc rises during OGD and the temporal relationship with mitochondrial ROS accumulation. A. Representative traces of Zn$^{2+}$ fluorescence (FluoZin-3, AM) in HeLa cells, showing the increases in [Zn$^{2+}$], during hypoxic treatment. Cells were continuously bathed in the hypoxic buffer throughout the fluorescence measurement as indicated by solid horizontal lines underneath all traces. There are two distinct Zn$^{2+}$ rises (Zn$^{2+}$ transient and Zn$^{2+}$ overload) and a latent phase between two elevations, which can be divided into four phases as depicted in C. A-1 is representative trace of OGD-induced Zn$^{2+}$ rises observed in 61% (n=33 in 6 separate experiments) of examined cells, where the first Zn$^{2+}$ increase or Zn$^{2+}$ transient does not return to the basal level with a short phase II and elevated phase III, which was caused by sustained increases in [Zn$^{2+}$], due to continuous OGD. Grey line trace represents cells treated with OGD buffer in the presence of Zn$^{2+}$ chelator TPEN. Dotted line traces (A-1&2) represent control cells with Zn$^{2+}$ fluorescence during OGD, under normoxic conditions. A-2 is representative of OGD-induced Zn$^{2+}$ increases observed in 39% of examined cells, where Zn$^{2+}$ transient returns to the basal level. B. Representative Zn$^{2+}$ fluorescent images of HeLa cells during OGD (FluoZin-3, AM), control is HeLa cells exposed to physiological buffer. Minute 0 is the baseline fluorescence before exposure to OGD, where the Zn$^{2+}$ fluorescence is very faint. At 4 minutes after OGD, there is a significant increase in fluorescence. At minute 8, the fluorescence is lower and remains low at minute 12, 16. The Zn$^{2+}$ fluorescence starts to rises at minute 20. Scale bar is 10µm. D. Representative traces of Zn$^{2+}$ (FluoZin-3, AM) and mitochondrial ROS (MitoSox Red) during OGD, plotted on the same graph to show temporal relationship of the two important phenomena. The lines above representative traces and the roman numerals indicate the phases of OGD-induced Zn$^{2+}$ rises.

**FIGURE 2.** Thapsigargin (TG) and FCCP sensitive intracellular sources of OGD-induced increases in [Zn$^{2+}$]. A. Average traces of Zn$^{2+}$ fluorescence during OGD treatment in HeLa cells with or without TG pretreatment (2µM). Black trace represents cells pretreated with TG for 20 minutes before the induction with OGD (TG + OGD), where the baseline is normalized to the elevated Zn$^{2+}$ before the OGD exposure (n = 12 in two separate experiments). Grey trace represents control (DMSO + OGD) cells pretreated with 0.1% DMSO before the induction of OGD (TG was dissolved in DMSO) (n = 15 cells in two separate experiments). The black line under the traces represents the duration of OGD treatment. Error bars are standard deviation. B. Average trace of Zn$^{2+}$ fluorescence (FluoZin-3, AM), showing FCCP-induced [Zn$^{2+}$] rises during normoxic condition (1µM FCCP, n=12 cells from two separate experiments), baseline is normalized to starting elevated Zn$^{2+}$ level. The black line under the traces represents the FCCP treatment. Error bars are standard deviation. C. Average traces of Zn$^{2+}$ fluorescence in HeLa cells that were pretreated with or without FCCP before OGD treatment. Black trace represents cells pretreated with FCCP for 10 minutes and washed out FCCP before OGD treatment (n = 20 from three separate experiments). Grey trace represents control cells pretreated with 0.1% DMSO before exposed to OGD (control n = 10 cells from two separate experiments). D. A bar graph showing a ratio of the peak fluorescence intensity of the second OGD-induced Zn$^{2+}$ rise divided by the peak fluorescence intensity of the first Zn$^{2+}$ rise. Error bars are representing a standard deviation (p value is 0.0017) (FCCP, n=20; control n=10).

**FIGURE 3.** Mitochondrial ROS production reduced by Zn$^{2+}$ chelation. A. Confocal images of a HeLa cell double labeled with Zn$^{2+}$ and mitochondrial ROS fluorescent indicators. Zinc: Zinpyr-1 (1µM) green fluorescence. Mito ROS: MitoSOX Red (5µM) red fluorescence. DIC: differential interference contrast image of the same cell by transmitted light to show overall structure of the cell. Merge: Composite.
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overlay fluorescence image. The yellow structures of the merged image show the co-localization of Zn\textsuperscript{2+} and mitochondrial ROS fluorescence. All images were captured at ×100. Scale bar -10μm. Arrows highlight a couple of mitochondria with co-localized signal. B. Average traces show the effect of TPEN, a Zn\textsuperscript{2+} specific chelator, on OGD-induced ROS productions in cells loaded with MitoSOX Red (5μM). Black line trace represents OGD-induced ROS accumulation (n = 15 from five separate experiments). Grey line trace represents OGD-induced ROS in the presence of TPEN (35μM) (n = 6 from two separate experiments). TPEN reduced OGD-induced ROS generation. The black line under the traces represents the hypoxic treatment. Dotted line trace represents control cells loaded with MitoSOX Red but without hypoxic treatment (n = 10 from five separate experiments). C. Average traces show the effect of TPEN, a Zn\textsuperscript{2+} specific chelator, on FCCP-induced ROS productions in cells loaded with MitoSOX Red (5μM), under normoxic condition. Black line trace represents FCCP (1μM)-induced ROS accumulation (n = 15 from two separate experiments). Grey line trace represents FCCP-induced ROS in the presence of TPEN (35μM) (n = 10 from two separate experiments). TPEN reduced FCCP-induced ROS generation. The black line under the traces represents the duration of FCCP treatment. Dotted line trace represents control cells loaded with MitoSOX Red but without FCCP treatment (n = 10 from five separate experiments).

FIGURE 4. Interplay between NADPH oxidase and Zn\textsuperscript{2+} in ROS accumulation, during induced intracellular Zn\textsuperscript{2+} increase. A. Bar graph of Zn\textsuperscript{2+}-induced mitochondrial ROS in HeLa cells loaded with ROS indicator MitoSOX Red. The fluorescence was measured at the end of 30 minutes of treatments with exogenous Zn\textsuperscript{2+} (n = 12 from two separate experiments), Zn\textsuperscript{2+} with apocynin, a NADPH oxidase inhibitor (apo, n = 12 from two separate experiments), or Zn\textsuperscript{2+} with oxypurinol, a xanthine oxidase inhibitor (oxy, n = 12 from two separate experiments). Zn\textsuperscript{2+} was co-applied with Zn\textsuperscript{2+} ionophore pyrithione (10μM), which induces rapid intracellular Zn\textsuperscript{2+} increase. ROS levels (mean ± SD) are expressed as percent change in mean fluorescence intensity from the baseline before treatment. Control is cells exposed to physiological buffer (n= 9 from three separate experiments), under normoxic conditions. B. A representative western blot of HeLa cells total cell homogenate, probed with anti-p47phox antibody, which detects endogenous levels of p47phox, a functional subunit of NADPH oxidase. In the first lane of the blot is control cells exposed to physiological buffer. The second lane is cells treated with sodium pyrithione (10μM) alone. The third lane is cells treated with combination of Zn\textsuperscript{2+} (50μM) and pyrithione (10μM), showing that intracellular Zn\textsuperscript{2+} increase leads to higher level of p47phox. The lower panel is the loading control, where the blot was stained with Coomassie Blue R-250 to show total protein on the blot after the probing with antibodies (n=2). C. Bar graph showing changes in baseline ROS fluorescence (HEt) in rat hippocampal slices after OGD and re-oxygenation. The fluorescence was measured at the end of 30 minutes of OGD and the end of 30 minutes of re-oxygenation. ROS levels (mean ± SD) are expressed as percent change of fluorescence intensity before the re-oxygenation and 30 minutes after re-oxygenation. Re-oxygenation caused a significant increase in HEt fluorescence (12.4% ± 1.1%, n=10 from two separate experiments). NADPH oxidase inhibitor apocynin (apo) reduced ROS accumulation to 4.6% ± 3.6%; TPEN, a Zn\textsuperscript{2+} chelator, caused greater reduction in ROS accumulation resulting in fluorescence -1.0 % ± 1.2%.

FIGURE 5. Summary of crosstalk between OGD-induced Zn\textsuperscript{2+} and ROS increases that involve intracellular Zn\textsuperscript{2+} storage, mitochondria and NADPH oxidase. A. Schematic drawing of intracellular Zn\textsuperscript{2+} storage including MT, organelle apparatus such as ER, and mitochondria, showing the release and uptake of Zn\textsuperscript{2+} by them in four described phases of Zn\textsuperscript{2+} rises during OGD. B. A diagram of temporal relationship of OGD-induced Zn\textsuperscript{2+} and ROS accumulation during the course of prolonged hypoxic exposure (30 minutes in the present study). Green area represents Zn\textsuperscript{2+}increases; red-line shows ROS accumulation. Roman numerals and vertical dashed lines show four phases of Zn\textsuperscript{2+}transient during OGD. C. Schematic drawing shows mitochondrial Zn\textsuperscript{2+} storage and release during OGD in relationship with mitochondrial ROS production and NADPH oxidase-mediated ROS production. a. Before OGD. b. Mitochondria uptake and store Zn\textsuperscript{2+} during OGD. Showing the positive feedback between mitochondrial Zn\textsuperscript{2+} stress facilitating ROS production which further releases Zn\textsuperscript{2+}. c. The large release of Zn\textsuperscript{2+} and ROS
from mitochondria. Zn$^{2+}$ may activate NADPH oxidase and consequently further increase ROS accumulation.
Figure 1

A-1

Zn$^{2+}$

- OGD
- normoxia
- OGD + TPEN

Time (min)

A-2

Zn$^{2+}$

- OGD
- normoxia

Time (min)

B.

OGD

normoxia

Time (min)

C. Zn$^{2+}$

I

II

III

IV

ROS

Zinc

ROS

OGD

Time (min)
Figure 2

A. $\text{Zn}^{2+}$

- DMSO + OGD
- TG + OGD

ΔF vs. Time (min)

B. $\text{Zn}^{2+}$ normoxia

- FCCP alone
- control

ΔF vs. Time (min)

C. $\text{Zn}^{2+}$

- DMSO + OGD
- FCCP + OGD

ΔF vs. Time (min)

D. Ratio of zinc 2nd increase to 1st peak

- FCCP (1μM) + OGD

*
Figure 3

A. 
- Zn$^{2+}$
- Mito ROS
- DIC
- Merge

B. 
- ROS
- OGD
- TPEN + OGD
- normoxia

C. 
- ROS normoxia
- FCCP
- FCCP+TPEN
- control
Figure 4

A. Zinc induced ROS

<table>
<thead>
<tr>
<th>Condition</th>
<th>Percent change fluorescence (%)</th>
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<td>Control</td>
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</tr>
<tr>
<td>apo (60μM)</td>
<td>30</td>
</tr>
<tr>
<td>oxy (60μM)</td>
<td>55</td>
</tr>
<tr>
<td>Zinc (50μM)</td>
<td>60</td>
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</tbody>
</table>

B. P47PHOX

- pyr: -
- Zn²⁺: -

Total protein

C. ROS

<table>
<thead>
<tr>
<th>Condition</th>
<th>Percent change fluorescence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>apo (180μM)</td>
<td>20</td>
</tr>
<tr>
<td>TPEN (35μM)</td>
<td>0</td>
</tr>
<tr>
<td>Re-oxygenation</td>
<td>-4</td>
</tr>
</tbody>
</table>
Figure 5

A. Time course of OGD (minutes)
- Zn\(^{2+}\) Release
- Zn\(^{2+}\) Uptake
- Latent
- Zn\(^{2+}\) Overload

B. OGD-induced zinc and ROS rises

C. State of cell viability
- Normal
- Injury/Death

Legend:
- Zinc
- ROS
- NADPH oxidase
- MT
- Organelle zinc stores
- Mitochondria